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Summary

Osteoporosis, resulting from progressive loss of structural bone during the period of egglaying in hens, is associated with an increased susceptibility to bone breakage. To study the genetic basis of bone strength, an F_2 cross was produced from lines of hens that had been divergently selected for bone index from a commercial pedigreed White Leghorn population. Quantitative trait loci (QTL) affecting the bone index and component traits of the index (tibiotarsal and humeral strength and keel radiographic density) were mapped using phenotypic data from 372 F_2 individuals in 32 F_1 families. Genotypes for 136 microsatellite markers in 27 linkage groups covering ~ 80% of the genome were analysed for association with phenotypes using within-family regression analyses. There was one significant QTL on chromosome 1 for bone index and the component traits of tibiotarsal and humeral breaking strength. Additive effects for tibiotarsal breaking strength represented 34% of the trait standard deviation and 7.6% of the phenotypic variance of the trait. These QTL for bone quality in poultry are directly relevant to commercial populations.

Introduction

Loss of structural bone or osteoporosis brings about weakening of the skeleton over the egg-laying period and is a major contributor to the high incidence of bone fractures that occurs in laying hens (Whitehead & Fleming 2000). It was previously suggested that almost a third of laying hens suffer from bone fractures at some point in their lives (Gregory & Wilkins 1989), and a recent report suggests that around 20% of laying hens suffer from bone breakage (Budgell & Silversides 2004). Osteoporosis in laying hens is brought about by a cessation of structural bone formation and a switch to medullary bone formation under the influence of oestrogen when the hens come into lay. Medullary bone is a weaker form of woven bone present mainly in cavities of the long bones where it provides a labile source of calcium for shell formation. However, during the laying period not only is medullary bone reabsorbed to contribute to shell formation, but structural bone is also mobilized so the net result is a progressive weakening of the skeleton (Whitehead & Fleming 2000; Whitehead 2004). Modern hybrid layers produce almost an egg per day for at least 6 months and therefore have a prolonged period of elevated oestrogen with ongoing re-absorption of structural bone. Environmental and dietary factors can ameliorate the symptoms of osteoporosis (Rennie et al. 1997; Fleming et al. 1998, 2003), but genetics can provide a permanent solution (Bishop et al. 2000).

Selective breeding for nine generations from a base population of commercial pedigreed White Leghorn hens has resulted in lines that clearly differ for bone strength (high vs. low). The improved bone strength in the high line is accompanied by a lower incidence of skeletal damage (Bishop et al. 2000). The selection procedure

used for generating these lines was based on retrospective selection of progeny for a bone index, which included tibiotarsal and humeral breaking strength and keel radiographic density measured post-mortem. While this selection index is not practical under commercial conditions, the lines allow identification of genetic markers associated with osteoporosis. Herein we describe the location of a QTL for bone quality using F_2 birds generated from the reciprocal cross of high- and low-bone index lines.

Materials and methods

F_2 population

Two White Leghorn lines, which had been divergently selected for nine generations on the basis of a high or low bone index from a commercial White Leghorn line (Bishop et al. 2000), were used for the creation of the population used in this study. Mean trait data on these lines can be found in Tables S1 and S2. The bone index contained positive weights for tibiotarsal and humeral strength and keel radiographic density, as well as a negative value for body weight. Twenty-five females and ten males per line were used to produce F_1 birds. Chicks were hatched in three batches and shipped to Roslin, UK where they were groupreared to 16 weeks under standard protocols. Retrospective selection was carried out on the basis of post-mortem analysis of the parent bones to identify four families whose parents showed the most extreme bone indices. Two males and eight females from each F_1 family were selected based on fertility for males and egg production for females. Each male was mated to two females from each of the other families, resulting in 32 families producing F_2 birds.

The F_2 birds were hatched in nine batches. Female offspring from families were retained for analysis. The pullets were group-reared under standard protocols to 16 weeks, when they were transferred to individual layer cages. Egg production was recorded for each hen, and measurements were made of egg biomechanical properties at 35 and 60 weeks of age. DNA was collected from F0 and F1 parents, as well as F_2 progeny. At 60 weeks of age, birds from families where there were at least 20 hens were killed, and bone characteristics were assessed to allow calculation of bone indices for each bird.

Phenotypic measurement

Bone-breaking strengths were determined by three-point destructive bending tests using a JJ Lloyd LRX50 materials testing machine running the software package NEXYGEN 2.0 (http://www.chatillon.com) and fitted with a 2500 N load cell. The bending jig consisted of two 10-mm diameter steel bar supports, 30 mm apart at the centre, and a 10-mm diameter cross head that approached at 30 mm/min. Bones were always oriented in the same plane. Breaking strength was defined as the maximum load in Newtons achieved during the test. The failure point was set at a load that was 30% of maximum. Egg biomechanics were determined as a 'compression to limit' test using the same machine as for bone biomechanics fitted with a 100 N load cell. The whole egg was placed on a flat plate and the head, which also consisted of a flat plate, approached at 20 mm/min. The preload was set at 0.5 N and the test was deemed to be complete when the upper plate had travelled 1 mm from the point at which the preload was reached. Breaking strength was defined as the maximum load in Newtons achieved during the test. For keel radiographic

density, the keel was excised and radiographed in a Faxitron 43855D soft X-ray apparatus (Quados Ltd, Camberley, UK) using Kodak MRE-1 high resolution mammography film in Min-R2 cassettes with a single Min-R intensifying screen. Exposure was for 15 seconds at 22 Kv. Each exposed plate included an aluminium step wedge for calibration. The films were digitized via a Panasonic WVBL600 monochrome video camera (with auto-gain control set to OFF) and a stabilized lightbox. Output from the camera was connected to a Macintosh computer fitted with a Data Translation DT2255 frame grabber card. Using the public-domain NIH IMAGE program (http://rsb.info.nih.gov/nih-image/), the steps from the step wedge were captured for each film and a short macro routine was written to calibrate each image quickly. This involved automatically moving a circular region of interest repeatedly over the step wedge in each image and measuring the mean pixel intensity within each region of interest. This produced a non-linear (3rd degree polynomial) calibration curve ($r^2 > 0.99$ for all exposures). Each keel was then delineated with the manual drawing tool and the mean pixel intensity, in mm of aluminium equivalent, was calculated within each keel boundary.

Genotyping

Samples of blood were collected by syringe from a wing vein, and DNA was prepared by standard procedures. A total of 103 microsatellite markers covering 26 autosomal and sex chromosome linkage groups were genotyped in F_0 and F_1 birds, as well as in 25% of the F_2 birds representing the top and bottom distributions for bone index. After initial analysis for potential QTL, the number of markers on four chromosomes (1, 3, 6 and 9) was increased, and the entire population of 393 animals was

genotyped for 136 informative markers (Table S3). Fragment sizes were determined using GENESCAN 3.1 DNA fragment analysis and GENOTYPER 2.1 (PE Biosystems, Foster City, USA). All pedigree information, marker genotypes and trait data were recorded in resSpecies (http://www.resspecies.org/). Map information can be viewed at http://www.thearkdb.org/arkdb/index/:jsp.

Analysis

Animals laying less than 230 eggs were omitted in the final analysis because periods of non-laying allow structural bone to be deposited. After parentage checking and genotyping edits, 372 hens were available for analysis. The QTL mapping method of Haley et al. (1994) was implemented using QTL EXPRESS (Seaton et al. 2002). Experiment-wide significance thresholds using all genotypes were estimated using 1000 iterations and were 8.1 for P = 0.05 and 9.8 for P = 0.01. The probability of one F_2 offspring having each of the four QTL genotypes (QQ, Qq, qQ and qq) at each position in the genome at 2-cM intervals was calculated conditionally upon the marker genotype. A linear model for the additive (a) and dominance effects (d) of a QTL at a given position was analysed by least squares for each trait where the additive effect was defined as half the difference between the two homozygotes and the dominance effect as the difference between the means of the heterozygotes and homozygotes.

Body weight and egg production in the three weeks prior to post-mortem were included as covariates, and hatch and family were included as fixed effects. Where appropriate, suggestive QTL loci were fitted as co-factors to account for their background genetic effects using backward elimination and substitution (Jansen

1993). The genetic length, including an arbitrary 20 cM for the end markers and for each linkage group with a single marker, was 3105 cM or about 80% of the consensus linkage map (Schmid et al. 2000). This includes the additional markers on chromosomes 1, 3, 6 and 9 genotyped across the whole population. The average marker interval was 22.8 cM, and the average polymorphic information content was 0.24 (range 0.004–0.803).

Results

F_0 and F_2 phenotypic means and variations, as well as F_2 phenotypic correlations, can be found in Tables S1 and S2.

Evidence for QTL and their effects

The evidence for a QTL affecting bone index on chromosome 1 (Fig. 1, Table 1) was strong ($P < 0.05$). This region also contained QTL for humeral and tibiotarsal breaking strength. The tibiotarsal breaking strength QTL was significant ($P < 0.01$) while the humeral breaking strength QTL approached significance ($P = 0.05$) (Fig. 1, Table 1). Chromosomal regions fitted as co-factors were on chromosome 5 at 14 cM and on chromosome 9 at 0 cM; however, the QTL for bone index and tibiotarsal breaking strength were significant at the 0.01 level whether or not these minor QTL were included in the model. No other QTL were detected in the analysis of the whole population, and the putative QTL on chromosomes 3, 6 and 9 were not confirmed.

Suggestive QTL for bone index and for keel radiographic density on chromosome 9 at 0 cM were found when QTL at 370 cM on chromosome 1 were fitted as a co-factor.

There was a suggestive QTL for bone index and humerus breaking strength at 14 cM on chromosome 5 with an F-statistic of 6.8 and 7.4 (QTL at 370 cM on chromosome 1 fitted as a co-factor).

The 95% confidence interval (CI) for the bone index QTL on chromosome 1 spanned a region of around 200 cM (Fig. 1, Table 1). The additive and dominance effects of the QTL are presented in Table 2. Standardized effects for tibiotarsal breaking strength were 34.3% of the trait standard deviation and 7.6% of the phenotypic variance (Table 2).

Discussion

A QTL region was identified on chromosome 1 at 370 cM (198-to-393-cM interval) which accounted for a large proportion of the difference between the founder strains for tibiotarsal (18%) and humeral breaking strength (27%) and the bone index. There was some indication that this QTL had a correlated effect on egg breaking strength at 60 weeks of age as evidenced by a non-significant QTL at 366 cM on chromosome 1. The QTL on chromosome 1 was not associated with keel radiographic density, although that trait was also part of the bone index. Underlying factors influencing long bone strength may differ from that of keel mineralization. For example, the keel is not subjected to the same compression forces and loads that are important in chickens for bone formation and remodelling, including changes in osteoclast activity (Reich et al. 2005). In addition the population variance for keel radiographic density (CV 15.8%) was less than for the tibiotarsal and humeral breaking strength (CV, 23.1 and 29.3%). Overall the lower rate of bone resorption present in the high line may be

related to lower numbers of osteoclasts (bone resorbing cells) observed in the high line (Fleming et al. 2006).

In another study using a broiler-layer cross, no significant QTL for bone quality traits were found (Schreiweis et al. 2005), but there was a suggestive QTL for humeral bone length and area on chromosome 1. However, these QTL were different from the predicted position of the QTL observed in this study, although the confidence intervals overlapped. In another study where extensive measurements of bone quality were made, no QTL were reported, but it should be noted that the founder animals (Jungle fowl and White Leghorns) did not differ for the traits (Jensen et al. 2005). In contrast to these studies that did not find QTL, we used F_0 birds with similar growth and egg production characteristics from a common genetic background that differed only in measures of bone quality. A number of studies in inbred mice and rats have reported QTLs for bone density traits that should be correlated with bone breakage (for a review, see Liu et al. 2003), but there are few QTL reported for breaking strength, which is a risk measurement of bone fracture. In a rare study of bone breaking strength measured in 655 inbred rats, five significant QTL were found, mostly with small effect (Li et al. 2002).

This study reports the first significant QTL related to bone quality in poultry. We expect the QTL alleles to be segregating in the pedigree population that is used currently to produce the Lohmann White Leghorn hens used for table egg production.

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Tab.1

Bone quality QTL on chicken chromosome 1 detected in females in an F2 reciprocal cross between divergent selection lines for bone index.

Trait	Position (cM)	95% CI1 (cM)	F statistic	Flanking markers	Position in Mb[2]
Tibiotarsal breaking strength	370	138-416	13.2	ADL0148-LEI0198	115.4–120.8
Humeral breaking strength	334	30-376	7.9	ADL0061-ADL0020	92.3–94.2
Bone index	370	198-393	9.3	ADL0148-LEI0195	115.4–120.8

1 Confidence interval.

2 Calculated from the position of flanking markers on the 2006 (galGal3) build (http://genome.ucsc.edu/cgi-bin/hgGateway).

Tab.2

Additive and dominance effects of bone quality QTL on chromosome 1 detected in females in an F_2 reciprocal cross between divergent selection lines for bone index.

Trait	Position (cM)	Additive ± SE	Dominance ± SE	Phenotypic variance(%)[1]	Standardized effect(% of trait SD)[2]
Tibiotarsal breaking strength	370	25.6 ± 5.0	-4.2 ± 7.6	7.6	34.3
Humeral breaking strength	334	9.82 ± 3.14	11.2 ± 4.70	4.8	21.4
Bone index	370	0.20 ± 0.04	-0.05 ± 0.07	2.7	29.2

1 Proportional decrease in the residual sums of squares by fitting the model with the QTL compared to the reduced model.

2 Standardized effect (the mean additive effect divided by the trait standard deviation).

Fig.1

Variance-ratio plot from the F_2 QTL analysis for bone index and tibiotarsal and humeral breaking strength on chicken chromosome 1. The thresholds for significant linkage at the 5% and 1% genomewise levels are indicated. Marker positions on the chromosome are represented by triangles.